



**EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 208 (FGE.208): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the , - unsaturation in ring / side - chain and precursors from chemical subgroup 2.2 of FGE.19 b y EFSA**

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## SCIENTIFIC OPINION

### Scientific Opinion on Flavouring Group Evaluation 208 (FGE.208): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the $\alpha,\beta$ -unsaturation in ring / side-chain and precursors from chemical subgroup 2.2 of FGE.19 by EFSA<sup>1</sup>

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#### ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of one flavouring substance from subgroup 2.2 of FGE.19 in the Flavouring Group Evaluation 208. The Flavour Industry has provided additional genotoxicity studies for *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. *p*-Mentha-1,8-dien-7-al will represent the other nine flavouring substances in FGE.208. Based on the presently available data the Panel concluded that some concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al remains. In order to clarify the genotoxic potential of this substance, the Panel considered that further *in vivo* testing should be performed. To address this, an *in vivo* Comet assay, considering the first site of contact (e.g. stomach or duodenum) and liver, should be carried out according to the Scientific Report of EFSA on Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports.

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#### KEY WORDS

FGE.19; subgroup 2.2, alicyclic aldehydes;  $\alpha,\beta$ -unsaturated

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## SUMMARY

Following a request from the European Commission the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to consider the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) evaluations of flavouring substances assessed since 2000, and to decide whether no further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000<sup>4</sup>. These flavouring substances are listed in the Register, which was adopted by Commission Decision 1999/217/EC and its consecutive amendments.

The present Flavouring Group Evaluation 208 (FGE.208), corresponding to subgroup 2.2 of FGE.19, concerns three alicyclic aldehydes with the  $\alpha,\beta$ -unsaturation in ring / side-chain and seven precursors for such. The  $\alpha,\beta$ -unsaturated aldehyde structure, which is a structural alerts for genotoxicity and the data on genotoxicity previously available for these 10 substances, did not rule out the concern for genotoxicity.

The Panel identified one flavouring substance, *p*-mentha-1,8-dien-7-al [FL-no: 05.117], among the 10 substances in the present FGE.19 subgroup 2.2, for which appropriate genotoxicity data could be used for reading across to the other substances in the subgroup; therefore genotoxicity data have been requested for *p*-mentha-1,8-dien-7-al [FL-no: 05.117] according to the test strategy worked out by the Panel.

The Flavour Industry has provided additional genotoxicity data for *p*-mentha-1,8-dien-7-al [FL-no: 05.117].

Based on the presently available data, the Panel concluded that some concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al [FL-no: 05.117] remains. In order to clarify the genotoxic potential of this substance, the Panel considered that further *in vivo* testing should be performed. To address this, an *in vivo* Comet assay, considering the first site of contact (e.g. stomach or duodenum) and liver, should be carried out according to the Scientific Report of EFSA on Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports.

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<sup>4</sup> Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. Official Journal of the European Communities 19.7.2000, L 180, 8-16.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The European Parliament and the Council Regulation (EC) No 2232/96<sup>5</sup> sets out the basic rules for the use of flavouring substances in or on foodstuffs in the EU. It furthermore lays down a Procedure for the establishment of a Community list of flavouring substances.

As a first step, the Procedure foresees the establishment of a register of the substances used in the Member States. The Register is laid down in the Commission Decision 1999/217/EC of 23 February 1999<sup>6</sup>, last amended by Commission Decision 2009/163/EC<sup>7</sup>.

Following the establishment of this Register, an evaluation programme was adopted by the Commission Regulation (EC) No 1565/2000 of 18 July 2000. Within five years of the adoption of the programme, in accordance with Regulation (EC) No 2232/96, a Community list of flavouring substances, the use of which is authorized to the exclusion of all others, must be drawn up.

In the 26<sup>th</sup> Plenary meeting of the AFC Panel on 27-29 November 2007, EFSA discussed the flavouring group evaluation 19 (FGE.19). FGE.19 contains those flavouring substances which are alpha, beta-unsaturated aldehydes or ketones and their precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation. The alpha, beta-unsaturated aldehyde and ketone structure is considered by the Panel to be a structural alert for genotoxicity. FGE.19 was divided into 28 subgroups. For subgroup 2.2, EFSA concluded that there is a need for additional information before conclusions on the substances in this subgroups can be reached.

The flavouring substances which belong to subgroup 2,2 are:

p-Mentha-1,8-dien-7-ol [FL-no: 02.060]

Myrtenol [FL-no: 02.091]

Myrtenal [FL-no: 05.106]

p-Mentha-1,8-dien-7-al [FL-no: 05.117]

2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121]

Myrtenyl formate [FL-no: 09.272]

p-Mentha-1,8-dien-7-yl acetate [FL-no: 09.278]

Myrtenyl acetate [FL-no: 09.302]

Myrtenyl-2-methylbutyrate [FL-no: 09.899]

Myrtenyl-3-methylbutyrate [FL-no: 09.900]

p-Mentha-1,8-dien-7-al [FL-no: 05.117] was selected as a representative substance for the subgroup 2.2 (The EFSA Journal (2008) 910, 1-5). The European Flavour and Fragrance Association has now submitted the information on genotoxicity studies for this representative substance.

<sup>5</sup> Regulation No 2232/96 of the European Parliament and of the Council of 28 October 1996. Official Journal of the European Communities 23.11.1996, L 299, 1-4.

<sup>6</sup> Commission Decision 1999/217/EC of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs. Official Journal of the European Communities 27.3.1999, L 84, 1-137.

<sup>7</sup> Commission Decision 2009/163/EC of 26 February 2009 amending Decision 1999/217/EC as regards the Register of flavouring substances used in or on foodstuffs. Official Journal of the European Union 27.2.2009, L 55, 41.

In order to be able to consider the substances in the subgroup 2.2 of FGE.19 for the initial Community list, we would ask EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances belonging to this subgroup.

#### **TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION**

The European Commission requests the European Food Safety Authority to carry out a risk assessment on *p*-mentha-1,8-dien-7-al [FL-no: 05.117] and on substances of subgroup 2.2 covered by [FL-no: 05.117] as a representative substance, as stated in “List of alpha,beta-unsaturated Aldehydes and Ketones Representative of FGE.19 Substances for Genotoxicity Testing (The EFSA Journal (2008) 910, 1-5), in accordance with Commission Regulation (EC) No 1565/2000.

## ASSESSMENT

### 1. History of evaluation

Regulation (EC) No 2232/96 of the European Parliament and the Council lays down a Procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC, as last amended by Commission Decision 2009/163/EC. Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000, which is broadly based on the opinion of the Scientific Committee on Food (SCF, 1999). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002<sup>8</sup>.

The Union list of flavourings and source materials is established in Commission Regulation (EC) No 872/2012<sup>9</sup>.

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being  $\alpha,\beta$ -unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and / or oxidation (EFSA, 2008a).

The  $\alpha,\beta$ -unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The  $\alpha,\beta$ -unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship ((Q)SAR) prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these  $\alpha,\beta$ -unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA,

<sup>8</sup> Commission Regulation No 622/2002 of 11 April 2002 establishing deadlines for the submission of information for the evaluation of chemically defined flavouring substances used in or on foodstuffs. Official Journal of the European Communities L 95, 10-11.

<sup>9</sup> Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Communities L 267, 1-161.

2008a) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established, FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225).

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavouring Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all, or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220, the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related  $\alpha,\beta$ -unsaturated substances in the different subgroups for which additional data are requested, EFSA has worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The present FGE.208 concerns the evaluation of the data requested on genotoxicity for subgroup 2.2 of FGE.19, which the Flavouring Industry has now submitted.

## **2. Presentation of the Substances in the Flavouring Group**

### **2.1. Description**

The present Flavouring Group Evaluation 208 (FGE.208), corresponding to subgroup 2.2 of FGE.19, concerns three alicyclic aldehydes with the  $\alpha,\beta$ -unsaturation in ring / side-chain and seven precursors for such aldehydes. The 10 substances under consideration in FGE.208 are listed in Table 2.

Eight of the flavouring substances have previously been evaluated by the JECFA (JECFA, 2002a). A summary of their current evaluation status by the JECFA and the outcome of this consideration is presented in Table 3.

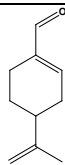
The  $\alpha,\beta$ -unsaturated aldehyde structure is a structural alerts for genotoxicity (EFSA, 2008a) and the data on genotoxicity previously available did not rule out the concern for genotoxicity for these 10 flavouring substances.

### **2.2. Representative Substance for Subgroup 2.2**

The Panel has identified one substance in subgroup 2.2 which will represent the other nine substances in this subgroup (EFSA, 2008c). For this substance genotoxicity data according to the test strategy (EFSA, 2008b) have been requested. The representative substance is shown in Table 1.



**Table 1:** Representative substance for subgroup 2.2 of FGE.19 (EFSA, 2008c)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no
05.117 973	p-Mentha-1,8-dien-7-al		3557 11788 2111-75-3

### 3. Additionally Submitted Genotoxicity Data on the Representative Substance of Subgroup 2.2

#### 3.1. Introduction

The Industry has submitted additional data concerning genotoxicity studies for the representative substance *p*-Mentha-1,8-dien-7-al [FL-no: 05.117] for this subgroup (EFFA, 2012). The data for *p*-mentha-1,8-dien-7-al are one *in vitro* test in bacteria and two *in vitro* tests in mammalian cell systems.

#### 3.2. *In vitro* Data

##### 3.2.1. Bacterial Reverse Mutation Assay

An Ames assay was conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of *p*-mentha-1,8-dien-7-al, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post mitochondrial fraction (S9-mix) in three experiments (Bowen, 2011). A batch of 93.1 % purity was used for the first and second experiment, while a batch of 91.9 % was used for the third experiment. An initial toxicity range finding experiment was carried out according to the plate incorporation method in the presence and absence of S9-mix for the TA100 strain only at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (solvent) and positive controls. Evidence of toxicity in the form of complete killing of the background lawn was observed at 5000 µg/plate in the absence and presence of S9-mix. Precipitation was also seen at this concentration. As valid mutation data were available from five different test concentrations, the data from these treatments were considered to be acceptable for mutation analysis as part of the first main experiment. This GLP study complies with OECD Guideline 471.

In the first experiment, treatments of all the remaining tester strains were performed in the absence and presence of S9-mix at concentrations of 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (solvent) and positive controls. Evidence of toxicity was observed in all strains in the absence and presence of S9-mix at 5000 µg/plate, and in some strains also at 1000 µg/plate. Precipitation was also seen at 5000 µg/plate. Valid mutation data were obtained from five or six different test concentrations in each strain. Following Experiment 1 treatments, a statistically significant and concentration related increase in revertant numbers was observed in strain TA98 at 200 (1.8-fold increase) and 1000 (3.2-fold increase µg/plate in the absence of S9-mix, when data were analysed at the 1 % level using Dunnett's test.

In a second experiment, treatments of the strains assayed in Experiment 1 were performed in the absence and presence of S9-mix at 8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate. Treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step

(60 minutes). Evidence of toxicity ranging from a marked reduction in revertant numbers and/or slight thinning of the bacterial lawn to a complete killing of the test bacteria was observed at 320, 800, and/or 2000 µg/plate and above in most of the strains in the absence and presence of S9-mix. Precipitation was again seen at 5000 µg/plate, particularly in the presence of S9-mix. However, valid mutation data were obtained from at least five test concentrations in each strain. Following Experiment 2 treatments, a statistically significant and concentration related increase in revertant numbers was again observed in strain TA98 in the absence of S9-mix at 320 (2.3-fold increase) and 800 (2.9-fold increase) µg/plate, when data were analysed at 1 % level using Dunnett's test.

Following the treatments in Experiments 1 and 2, *p*-mentha-1,8-dien-7-al increased the frequency of revertants in strain TA98 by at least 2-fold in the absence of S9-mix activation. These results were in contrast with what had been observed for *p*-mentha-1,8-dien-7-al in previous Ames assays described further below. One possible explanation for the varying pattern of behaviour was that the material tested (93.1 % purity) in Experiments 1 and 2, due to minor impurities, gave positive results. On this basis, a third experiment, which used a different batch (91.9 % purity) of test article but the same treatment conditions as in Experiment 1, was conducted in strain TA98. In the absence of S9-mix toxicity was observed at 5000 µg/plate, while in the presence of S9-mix toxicity was observed at all concentrations tested. Additionally, while precipitation was observed on all test plates at 5000 µg/plate in Experiments 1 and 2, no precipitation was observed at this concentration in Experiment 3. Following the treatments in Experiment 3, statistically significant and concentration related increases in revertant numbers for strain TA98 in the absence of S9-mix were observed at 8 µg/plate and above when the data were analysed at 1 % level using Dunnett's test. Therefore, the increases observed in strain TA98 were reproduced and are considered to be evidence of mutagenic activity in this strain. No other increases in revertant numbers were observed in all other strains that were statistically significant when the data were analysed at the 1 % level using Dunnett's test.

### 3.2.2. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) Assay

To assess mutagenic potential in a mammalian system, eukaryotic mouse lymphoma L5178Y cells were treated with *p*-mentha-1,8-dien-7-al in the absence and presence of S9-mix to study the induction of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus (Lloyd, 2012). A batch of 92.5 % purity was used. Across 3 different experiments, treatments were carried out for 3 hours in the absence of S9-mix, 3 hours in the presence of S9-mix and 24 hours in the absence of S9-mix, and each treatment regime was independently repeated. Concentrations for the main experiments were established in preliminary range-finding cytotoxicity experiments. This GLP study complies with OECD Guideline 476.

In the first mutation experiment, cells were treated with *p*-mentha-1,8-dien-7-al for 3 hours at 10, 20, 40, 60, 70, 80, 90 and 100 µg/ml in the absence of S9-mix and at 40, 60, 80, 100, 120, 140, 160 and 180 µg/ml in the presence of S9-mix. Percent relative survival (% RS) decreased to 13 % at 100 µg/mL in the absence of S9-mix and to 16 % at 180 µg/mL in the presence of S9-mix. Negative control mutant frequencies were normal, and were significantly increased by treatment with the positive control. No significant increases in mutation frequency were observed at any concentration analysed in the presence or absence of S9-mix in this experiment, and no statistically significant linear trends were observed.

In a second experiment, cultures were treated with *p*-mentha-1,8-dien-7-al for 3 hours at 20, 40, 50, 60, 70, 80, 90, 100 and 120 µg/ml in the absence of S9-mix and at 25, 50, 75, 100, 120, 140, 160, 170 and 180 µg/ml in the presence of S9-mix. Percent RS decreased to 7 % at 120 µg/mL in the absence and to 10 % at 180 µg/mL in the presence of S9-mix. Also in this experiment, 24-hour treatments were carried out with *p*-mentha-1,8-dien-7-al in the absence of S9-mix at 4, 8, 12, 15, 18 and 21 µg/mL of *p*-mentha-1,8-dien-7-al. Percent RS decreased to 9 % at the highest concentration. Negative control mutant frequencies were normal and were significantly increased by treatment with the

positive control. In the absence and presence of S9-mix, there were no statistically significant increases in mutant frequency relative to control at any concentration analysed, although in the absence of S9-mix (both 3- and 24-hour treatments), there were statistically significant linear trends.

In a third experiment, cultures were treated with *p*-mentha-1,8-dien-7-al for 24 hours at 4, 8, 12, 14, 16, 18 and 20 µg/ml in the absence of S9-mix. Percent RS decreased to 14 % at the highest concentration. Negative control mutant frequencies were normal, and were significantly increased by treatment with the positive control. There were no significant or dose-related increases in mutant frequency following *p*-mentha-1,8-dien-7-al treatments. The observations made with the 24-hour treatments in the second experiment were not reproduced at similar concentrations and extents of toxicity and were considered not to be biologically relevant by the authors (Lloyd, 2012).

However, it is not clear why the 3-hour treatment was not repeated. Overall, the results in the HPRT assay in the absence of S9-mix should be considered, differently from the authors opinion, as equivocal instead of negative, based on the statistically significant trends in both 3- and 24-hour treatments in the second experiment.

### 3.2.3. *In vitro* Micronucleus Assays

A batch of 94.9 % purity of *p*-mentha-1,8-dien-7-al was assayed for the induction of chromosome damage in mammalian cells *in vitro* by examining its effect on the frequency of micronuclei (MN) in cultured human peripheral blood lymphocytes (whole blood cultures pooled from 2 healthy male volunteers) treated in the absence and presence of S9-mix (Lloyd, 2009). *p*-Mentha-1,8-dien-7-al was added at 48 hours following culture initiation (stimulation by phytohaemagglutinin) either for 3 hours in the absence or presence of S9-mix followed by 21 hours recovery, or for 24 hours in the absence of S9-mix. Cytochalasin B (6 µg/ml) was added either at the start of treatment (24-hour treatments) or at the start of recovery (following 3 hours treatments) in order to block cytokinesis and generate binucleate cells for analysis. It remained in the cultures until they were harvested 24 hours after the start of treatment. A range-finding experiment had been conducted with and without S9-mix treatment in order to provide toxicity information (reduction in replication index, RI) that could be used as a basis for choosing a range of concentrations to be evaluated in the main micronucleus analysis.

In the main assay, micronuclei were analysed from at least three concentrations for each treatment condition. For 3-hour treatment without S9-mix the concentrations were 80, 100, 110 and 120 µg/ml, for 3-hour treatment with S9-mix the concentrations were 100, 120 and 140 µg/ml and for 24-hour treatment without S9-mix the concentrations were 20, 25 and 35 µg/ml. The levels of cytotoxicity (reduction in RI) at the top concentrations reached 58 and 45 % in the 3-hour treatment in the absence and presence of S9-mix and 58 % in the 24-hour treatment in the absence of S9-mix, respectively. These levels of cytotoxicity therefore reached, or were very close to, the recommended (50 - 60 %) range of cytotoxicity. One thousand binucleate cells per culture from 2 replicate cultures per concentration were scored for micronuclei. This GLP study complies with OECD Test Guideline 487.

The frequencies of micronucleated binucleate (MNBN) cells in negative control cultures were normal, and were significantly increased by treatment with positive control chemicals. Treatment of cells with *p*-mentha-1,8-dien-7-al in the absence and presence of S9-mix under all treatment conditions resulted in frequencies of MNBN cells that were similar to and not significantly different from those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all *p*-mentha-1,8-dien-7-al treated cultures fell within (or slightly below) normal ranges. It was concluded that *p*-mentha-1,8-dien-7-al did not induce micronuclei in cultured human peripheral blood lymphocytes when tested at toxic concentrations in both the absence and presence of S9-mix (Lloyd, 2009).

## 4. Previously Available Data

### 4.1. *In vitro* Data

Several *In vitro* mutagenicity/genotoxicity tests have been performed on the FGE.19 Subgroup 2.2 Representative Substance *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. The quality of most of them could not be adequately evaluated, either because they are in Japanese and therefore details are difficult to obtain or because of limitations in the experimental design. Negative results were reported by Ishidate *et al.* (1984) for an Ames test in which *Salmonella typhimurium* strains TA92, TA1535, TA100, TA1537, TA94 and TA98 were used. Duplicate plates were used for each of six concentrations up to 1000 µg/plate with S9-mix. The sample used had the same purity (93.1 %) of the batch used by Bowen (2011). The results were only reported as – or + (a + would be given if revertant numbers exceeded 2x concurrent control) and therefore weaker responses may have been observed but cannot be verified. Fujita *et al.* (Fujita *et al.*, 1994) also reported negative results for an Ames assay in strains TA97 and TA102 performed both with and without S9-mix. The top concentration of *p*-mentha-1,8-dien-7-al was less than in the Ishidate study, namely 100 µg/plate. Negative results were reported in mutation tests in which *p*-mentha-1,8-dien-7-al was incubated with *Escherichia coli* WP2 cells at 50 to 400 µg/plate (Yoo, 1986). Few details can be obtained from the paper, but it appears that the maximum increase in revertants was 1.3-fold, which is considered negative. However, only one result was given, so the test was probably only conducted in the absence of S9-mix.

*p*-Mentha-1,8-dien-7-al was considered to be weakly positive in the *rec*-assay with *Bacillus subtilis* strains M45 and H17 at a concentration of 2.5 µl *p*-mentha-1,8-dien-7-al/disk, probably equivalent to 2500 µg/disk (Yoo, 1986). This study is a very short paper, with very few details. Another study using the same strains reported negative results for *p*-mentha-1,8-dien-7-al at concentrations between 0.16 and 0.63 µL/plate (corresponding to 0.15 and 0.6 µg/plate) and positive results at higher concentrations of 1.25 and 2.5 µL/plate (1.2 and 2.4 µg/plate) (Kuroda *et al.*, 1984). It should be noted that these DNA damage assays in bacteria do not detect mutation, are non-standard and not requested by regulatory agencies. The results cannot therefore be considered to carry as much weight as results from recommended standard assays.

In a study by Eder *et al.*, 1993 *p*-mentha-1,8-dien-7-al gave negative results in a SOS-Chromotest with genetically engineered *Escherichia coli*. The maximum induction factor ( $I_{max}$ ) with *p*-mentha-1,8-dien-7-al was calculated to be 1.0. Positive results are considered to be significant if the  $I_{max}$  is at least 1.5. The SOS-Chromotest is also not a mutation test. It measures induction of the SOS repair system, and this is interpreted as indicating DNA damage. The results cannot therefore be considered to carry as much weight as results from recommended standard assays.

Standard chromosomal aberration (CA) assays for *p*-mentha-1,8-dien-7-al have yielded positive results. In a CA study by Ishidate *et al.* (Ishidate *et al.*, 1984), Chinese hamster fibroblasts (CHL) were only treated in the absence of S9-mix for 24 or 48 hours with a batch of 93.1 % purity. There were no treatments in the presence of S9-mix. Concentrations for the main CA test were selected from a preliminary experiment in which cell density (a crude and subjective measure) on the culture dishes was assessed, but there was no concurrent measure of cytotoxicity in the CA test. Only single cultures of CHL cells were treated with each of 3 concentrations, and therefore only 100 cells/concentration were scored for CA. CA (including gaps) frequencies of 4.9 % or less were considered negative, 5.0–9.9 % were equivocal, and 10 % or higher were considered positive. *p*-Mentha-1,8-dien-7-al gave a strong positive response (39 % cells with CA and also an increase in polyploid cells to 31 %) at 50 µg/ml. In particular, structural chromosome aberrations were detected at 40 µg/ml at 24 hours (20.0 %) and at 48 hours (28.0 %); the strongest effect was observed at 50 µg/ml at 24 hours. An increase in polyploidy cells was also detected at 40 µg/ml (15 %) and 50 µg/ml (31 %) after 48 hours. As there was no concurrent measure of cytotoxicity, and the results at the other concentrations tested were not given, these results should be considered with caution; however, they cannot be completely dismissed. In the CA study of Tayama *et al.* (Tayama *et al.*, 1990) in CHO-K1 cells, a significant

increase in CA at 150 µg/ml in the absence of S9-mix was associated with no detectable cell division. This result can probably be dismissed as likely to be an artefact of high levels of cell killing. However, a significant increase in CA at 300 µg/ml in the presence of S9-mix was associated with 62 % proliferating cells, which does not indicate excessive toxicity. Most of the chromosome aberrations were chromatid exchanges. These results are clearly in contrast to the negative micronucleus results obtained in human lymphocytes in the recent GLP study (Lloyd, 2009). The reasons of such discrepancy are unclear.

A sister chromatid exchange (SCE) assay was performed with and without metabolic activation in CHO-K1 cells at concentrations up to 300 µg *p*-mentha-1,8-dien-7-al/ml (Tayama et al., 1990). Cytotoxicity was determined by the percentage of cells that showed differentially stained chromatids, i.e. had divided. A doubling of SCE/cell would usually be considered biologically relevant, and in the absence of S9-mix there was a doubling of SCE/cell at 150 µg/ml, where there was little toxicity, whereas in the presence of S9-mix there was a doubling of SCE/cell at all concentrations from 100-300 µg/ml, where there was low or moderate toxicity. However, SCE assays also only provide limited information for assessment of genotoxicity. The mechanism of induction of SCE, and its relevance for mutation and cancer is not understood.

Studies for induction of ouabain resistant mutants conducted in human fetus cells (Rsa) at concentrations of 0, 0.010, 0.015, 0.020 or 0.025 µg/ml gave negative results for *p*-mentha-1,8-dien-7-al at the lowest concentration, positive results (8 - 16 fold increases) for concentrations ranging from 0.015 to 0.02 µg/ml (where toxicity was slight to moderate), and showed *p*-mentha-1,8-dien-7-al to be cytotoxic at the highest concentration (Suzuki et al., 1990). In another mutagenicity study with Rsa cells (Suzuki and Suzuki, 1994), induction of ouabain resistance was reported at concentrations of > 10 ng *p*-mentha-1,8-dien-7-al/ml with apparent cytotoxicity at 20 ng/ml or higher. Also in this study, mutagenicity was detected (K-ras codons) at concentrations of 2 - 200 ng/ml. Human fetal (Rsa) cells are not routinely used for genotoxicity testing, so evaluation of the quality of the data is difficult. The concentrations used in these tests are much lower than in other mammalian cell tests, and possible reasons for the discrepancy are not clear. Sasaki *et al.* (Sasaki et al., 1990) tested *p*-mentha-1,8-dien-7-al for induction of ouabain-resistant mutants in CHO-K1 cells. The mutant frequency at the only concentration of *p*-mentha-1,8-dien-7-al tested (10 µg/ml, which reduced survival to 83.5 % of controls) appears to be low (0.7 mutants per 10<sup>6</sup> cells, compared to zero in controls) and the result would probably be considered negative. The study of ouabain resistance in all of these studies makes interpretation difficult. Ouabain resistance is generally considered not to be a sensitive mutagenic target (spontaneous frequencies very low; frame-shift mutations not detected), and it is difficult to conclude negative results when there is a zero incidence of effects in controls. The biological significance of large increase in ouabain resistant mutants at very low concentrations is equally difficult to interpret. This endpoint is no longer used in regulatory testing.

#### 4.2. *In vivo* Data

*In vivo* mutagenicity/genotoxicity testing has been performed on the FGE.19 Subgroup 2.2 Representative Substance *p*-mentha-1,8-dien-7-al (Table 5). Eight-week-old male ddY mice were administered a single intraperitoneal injection of *p*-mentha-1,8-dien-7-al [FL-no: 05.117] at doses of 75, 150, 300 or 600 mg/kg bw for a mouse micronucleus assay (six mice/group). The dosing regimen and the maximum dose was based on a pilot experiment with 2 mice/group. In the main experiment, after 24 hours the mice were killed and femoral bone marrow cells were collected, fixed and stained with Giemsa. One thousand polychromatic erythrocytes were scored per mouse. No indication of micronucleus induction was reported at any dose level (Hayashi et al., 1988). However, the study does not comply with current guidelines, because, after a single administration, groups of animals should be sacrificed 24 and 48 hours later. Also only 1000 PCE were scored per animal whereas the current recommendation is for 2000 PCE/animal.



## 5. Discussion

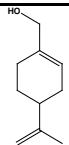
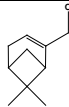
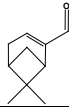
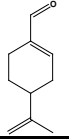
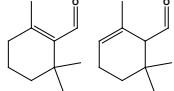
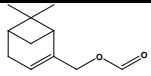
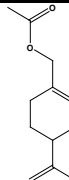
EFFA has submitted three valid, new *in vitro* studies, one in bacteria (Ames test) and two in mammalian cells (MN in human lymphocytes, HPRT in mouse lymphoma cells). The Ames test resulted positive, in the absence of metabolic activation with strain TA98, able to detect gene mutations of frameshift type (insertions/deletions). Equivocal results were reported in the HPRT assay (negative according to the authors) and negative results were reported in the MN test. Equivocal or negative results in the HPRT assay cannot dismiss the positive findings in the new Ames test, positive in the TA98 strain. The different results may be due to a different sensitivity of the two tests to detect frameshift mutations. In this respect, the Panel noted that the molecular analysis of mutational spectra at the *hprt* locus show a prevalence of GC to AT transitions and AT to CG transversions among spontaneous mutants, with less than 10 % of frameshifts (Chen et al, Environ Molec Mutagen 39: 296-305, 2002). Thus, given the prevailing contribution of mutations different from frameshift to the baseline incidence of *hprt* mutant colonies, it is expected that a many-fold increase in frameshift mutations is needed to give raise to an overall increase in mutation frequency which is detectable and significant on statistical grounds. The Ames test is generally considered as the most sensitive *in vitro* test for the prediction of genotoxic carcinogens and “false positive results” are rare; in this case, the positivity in the TA98 cannot be considered as a “false positive” without any explanation.

Negativity in mammalian cells “per se” cannot be considered more relevant than positivity in bacteria, simply on the basis of the complexity of cells. Among the previously supplied data, several *in vitro* and one *in vivo* mutagenicity/genotoxicity published studies are available. For most of them, performed not in compliance with current guidelines, the quality of data was limited. Negative results were reported in a study with the Ames test; however, the results were only reported as + or - , and therefore could not be verified. Both positive and negative results were reported for induction of ouabain gene mutations in mammalian cells, in limited studies. Ouabain resistance is generally considered of low sensitivity, compared with other gene mutation assays and is unable to detect mutations of frameshift type; it is no longer routinely used for regulatory purposes. Strong clastogenic effects in the absence of S9-mix were reported in Chinese hamster cell lines in two papers. Notwithstanding some limitations of the study, this positive results cannot be completely dismissed by the negative results in the new *in vitro* MN assay. The different types of cells used (Chinese hamster cell lines and human lymphocytes) and the different concentrations used can only partially explain the different results, which remain unclear. Negative results were reported in a mouse MN assay, in a study of limited validity for inadequate experimental design and insufficient presentation of data. Other published results, both positive and negative for DNA-damage/repair (rec-assay) in bacteria, negative for SOS and positive for SCE in mammalian cells, are not considered as relevant for the assessment of the genotoxic potential of *p*-mentha-1,8-dien-7-al.

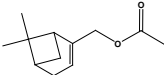
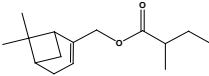
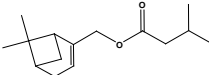
## CONCLUSIONS

Overall, the presently available data raise some concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. In order to clarify the genotoxic potential of this substance, the Panel considered that further *in vivo* testing should be performed. To address this, an *in vivo* Comet assay, considering the first site of contact (e.g. stomach or duodenum) and liver, should be carried out according to the Scientific Report of EFSA on Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports (EFSA, 2012).

**Table 2:** Specification Summary of the Substances in FGE.208 (JECFA, 2002b)

Table 2: Specification Summary of the Substances in the Present Group (JECFA, 2002b)							
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
02.060 974	p-Mentha-1,8-dien-7-ol		2664 2024 536-59-4	Liquid C <sub>10</sub> H <sub>16</sub> O 152.24	Slightly soluble Miscible	119 (14 hPa) NMR 96 %	1.495-1.505 0.956-0.963
02.091 981	Myrtenol		3439 10285 515-00-4	Liquid C <sub>10</sub> H <sub>16</sub> O 152.24	Insoluble Miscible	221 IR NMR 95 %	1.490-1.500 0.976-0.983
05.106 980	Myrtenal		3395 10379 564-94-3	Liquid C <sub>10</sub> H <sub>14</sub> O 150.22	Insoluble Miscible	220 NMR 98 %	1.496-1.507 0.984-0.990
05.117 973	p-Mentha-1,8-dien-7-al		3557 11788 2111-75-3	Liquid C <sub>10</sub> H <sub>14</sub> O 150.22	Insoluble Miscible	104 (13 hPa) NMR 97 %	1.504-1.513 0.948-0.956
05.121 979	2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde		3639 2133 432-25-7	Liquid C <sub>10</sub> H <sub>16</sub> O 152.23	Insoluble Miscible	62 (4 hPa) IR 99 %	1.476-1.483 0.950-0.957
09.272 983	Myrtenyl formate		3405 10858 72928-52-0	Liquid C <sub>11</sub> H <sub>18</sub> O <sub>2</sub> 180.25	Insoluble Miscible	127-130 (52hPa) NMR 96 %	1.477-1.483 1.004-1.010 (20°)
09.278 975	p-Mentha-1,8-dien-7-yl acetate		3561 10742 15111-96-3	Liquid C <sub>12</sub> H <sub>18</sub> O <sub>2</sub> 194.27	Insoluble Miscible	218-223 NMR 97 %	1.476-1.487 0.972-0.980

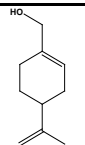
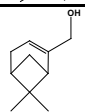
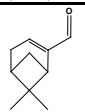
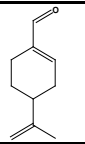
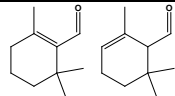
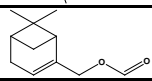
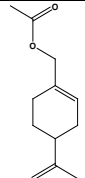
**Table 2: Specification Summary of the Substances in the Present Group (JECFA, 2002b)**

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
09.302 982	Myrtenyl acetate		3765 10887 1079-01-2	Liquid $C_{12}H_{18}O_2$ 194.28	Miscible	134 (49 hPa) IR NMR MS 98 %	1.470-1.477 0.987-0.996
09.899	Myrtenyl-2-methylbutyrate		138530-44-6	Liquid $C_{15}H_{24}O_2$ 236.35	Practically insoluble or insoluble Freely soluble	345 MS 95 %	1.466-1.470 0.964-0.970
09.900	Myrtenyl-3-methylbutyrate		33900-84-4	Liquid $C_{15}H_{24}O_2$ 236.35	Practically insoluble or insoluble Freely soluble	98 (1 hPa) MS 95 %	1.470-1.476 0.967-0.973

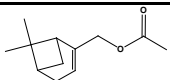
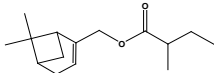
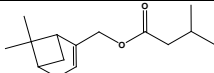
- 1) Solubility in water, if not otherwise stated.
- 2) Solubility in 95 % ethanol, if not otherwise stated.
- 3) At 1013.25 hPa, if not otherwise stated.
- 4) At 20°C, if not otherwise stated.
- 5) At 25°C, if not otherwise stated.



**Table 3:** Current Safety Evaluation Status Applying the Procedure (Based on Intakes Calculated by the MSDI Approach) (JECFA, 2002a)

Table 3: Summary of Safety Evaluation of the JECFA Substances in the Present Group (JECFA, 2002a)						
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI ( $\mu\text{g/capita/day}$ )	Class 2) Evaluation procedure path 3)	JECFA Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (genotoxicity)
02.060 974	p-Mentha-1,8-dien-7-ol		1.6 1	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
02.091 981	Myrtenol		0.37 0.03	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
05.106 980	Myrtenal		4.0 7	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
05.117 973	p-Mentha-1,8-dien-7-al		2.1 2	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
05.121 979	2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde		0.37 ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
09.272 983	Myrtenyl formate		0.3 ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
09.278 975	p-Mentha-1,8-dien-7-yl acetate		0.35 0.07	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.

**Table 3: Summary of Safety Evaluation of the JECFA Substances in the Present Group (JECFA, 2002a)**

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI ( $\mu\text{g/capita/day}$ )	Class 2) Evaluation procedure path 3)	JECFA Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (genotoxicity)
09.302 982	Myrtenyl acetate		0.37 0.04	Class I A3: Intake below threshold	4)	Evaluated in FGE.208, additional genotoxicity data required.
09.899	Myrtenyl-2-methylbutyrate		0.012	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.208, additional genotoxicity data required.
09.900	Myrtenyl-3-methylbutyrate		0.061	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.208, additional genotoxicity data required.

1) EU MSDI: Amount added to food as flavour in (kg / year)  $\times 10^9$  / (0.1  $\times$  population in Europe (= 375  $\times 10^6$ )  $\times 0.6 \times 365$ ) =  $\mu\text{g/capita/day}$ .

2) Thresholds of concern: Class I = 1800  $\mu\text{g/person/day}$ , Class II = 540  $\mu\text{g/person/day}$ , Class III = 90  $\mu\text{g/person/day}$ .

3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

4) No safety concern based on intake calculated by the MSDI approach of the named compound.

5) Data must be available on the substance or closely related substances to perform a safety evaluation.

ND Not determined

**Table 4:** Genotoxicity (*in vitro*)

**Table 4: Summary of Submitted *in vitro* Genotoxicity Data on the Representative Substance of Subgroup 2.2**

Chemical name [FL-no]	Test system	Test object	Concentration	Results	Comments	Reference
p-Mentha-1,8-dien-7-al [05.117]	Reverse mutation	<i>S. typhimurium</i> TA100	1.6, 8, 40, 200, 1000 and 5000 µg/plate	Negative <sup>b</sup>	Reliable without restriction. GLP study in compliance with OECD Guideline 471.	(Bowen, 2011)
		<i>S. typhimurium</i> TA98, TA102, TA1535, TA1537	0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Positive <sup>b</sup>	All strains were negative except TA98 without S9-mix treatment.	
		<i>S. typhimurium</i> TA98, TA102, TA1535, TA1537	8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate	Positive <sup>a, c</sup>	All strains were negative except TA98 without S9-mix treatment.	
		<i>S. typhimurium</i> TA98	0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Positive <sup>b</sup>	A different batch of test article was used and positive results in TA98 without S9- mix were confirmed.	
	Reverse mutation	<i>S. typhimurium</i> TA97, TA102	Up to 100 µg/plate	Negative <sup>b</sup>	Not assignable. Low concentrations; only two strains used, one of which (TA97) not routinely used.	(Fujita et al., 1994)
	Reverse mutation	<i>S. typhimurium</i> TA92, TA1535, TA100, TA1537, TA94, TA98,	Up to 1000 µg/plate	Negative <sup>a</sup>	Reliable with restriction. Results reported as - or +.	(Ishidate et al., 1984)
	Mutagenicity	<i>E. coli</i> WP2	Up to 0.4 mg/plate	Negative <sup>c</sup>	Not assignable. Probably only performed in the absence of S9-mix. Low concentrations tested; only few details available.	(Yoo, 1986)
	DNA damage	<i>B. subtilis</i> M45 and H17	2.5 µl/disk (probably equivalent to 2500 µg/disk)	Weak positive	Not assignable. Details difficult to obtain. Endpoint not relevant.	(Kuroda et al., 1984)
	DNA damage	<i>B. subtilis</i> M45 and H17	0.16-0.63 µL/plate (0.15-0.6 µg/plate) 1.25 and 2.5 µL/plate (1.2 and 2.4 µg/plate)	Negative Positive		
	DNA repair	<i>E. coli</i> PQ37	Not reported	Negative	SOS Chromotest. Endpoint not relevant.	(Eder et al., 1993)
	Sister chromatid exchange	Chinese hamster ovary cells	150 µg/ml	Positive <sup>c</sup>	Reliable with restriction; genetic endpoint of limited relevance.	(Tayama et al., 1990)
			100-300 µg/ml	Positive <sup>e</sup>		
	Chromosomal aberration	Chinese hamster fibroblasts	Up to 50 µg/ml	Positive <sup>c</sup>	Reliable with restriction. No concurrent measure of cytotoxicity. Performed only in the absence of S9.	(Ishidate et al., 1984)
	Chromosomal aberration	Chinese hamster ovary cells	300 µg/ml	Positive <sup>e, f</sup>	Reliable with restriction. Moderate toxicity at 300 µg/ml (+S9).	(Tayama et al., 1990)

**Table 4: Summary of Submitted *in vitro* Genotoxicity Data on the Representative Substance of Subgroup 2.2**

Chemical name [FL-no]	Test system	Test object	Concentration	Results	Comments	Reference
			150 µg/ml	Negative <sup>c, d, f</sup>	Reliable with restriction. No detectable cell division at 150 µg/ml (-S9).	
	Mutagenicity	Chinese hamster ovary cells	10 µg/ml	Negative <sup>c, g</sup>	Not assignable. Ouabain resistance measured. Only one concentration tested without S9; insufficient details.	(Sasaki et al., 1990)
	Mutagenicity	Human fetus cells (Rsa)	Up to 0.025 µg/ml	Positive <sup>h</sup>	Unreliable; ouabain resistance measured in Rsa cells not routinely used; insufficient details.	(Suzuki et al., 1990)
			0.010 µg/ml	Negative <sup>h</sup>		
	Mutagenicity	Human fetus cells (Rsa)	>10 ng/ml	Positive <sup>i</sup>	Japanese paper quoted but not available	(Suzuki and Suzuki, 1994)
	Micronucleus Induction	Primary human lymphocytes	Up to 140 µg/ml <sup>l</sup>	Negative <sup>b</sup>	Reliable without restriction. Complies with GLP and OECD 487 Guideline.	(Lloyd, 2009)
	HPRT assay	Mouse lymphoma L5178Y cells	Up to 180 µg/ml <sup>k</sup>	Equivocal <sup>b</sup>	Reliable without restriction. Complies with GLP and OECD 476 Guideline	(Lloyd, 2012)

a Preincubation with exogenous metabolic system from rat liver.

b Assay performed with and without metabolic activation.

c Assay performed without metabolic activation.

d Cytotoxic at 150 µg/ml.

e Assay performed with metabolic activation.

f Positive only at cytotoxic concentrations.

g Cytotoxic at 12 µg/ml.

h Cytotoxic at 0.025 µg/ml.

i Cytotoxic at > 20 ng/ml.

j Cytotoxic ≥ 160 µg/ml.

k Cytotoxic ≥ 180 µg/ml (3 hour treatment in the presence of S9); cytotoxic ≥ 100 µg/ml (3 hour treatment in the absence of S9); cytotoxic ≥ 21 µg/ml (24 hour treatment in the absence of S9).

**Table 5:** Genotoxicity (*in vivo*)

<b>Table 5: Summary of Additionally Submitted <i>In Vivo</i> Genotoxicity Data on the representative substance of subgroup 2.2</b>							
<b>Chemical Name FL-no</b>	<b>Test System <i>in vivo</i></b>	<b>Test Object</b>	<b>Route</b>	<b>Dose</b>	<b>Result</b>	<b>Reference</b>	<b>Comments</b>
p-Mentha-1,8-dien-7-al [05.117]	Micronucleus Assay	Mouse bone marrow cells	Intraperitoneal	75, 150, 300 or 600 mg/kg bw	Negative	(Hayashi et al., 1988)	Unreliable; sampling time only at 24 hours; only 1000 PCE per animal scored; poor presentation of data.

## REFERENCES

- Benigni R and Netzeva T, 2007a. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated aldehydes in *S. typhimurium* TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
- Benigni R and Netzeva T, 2007b. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated ketones in *S. typhimurium* TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
- Bowen R, 2011. Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. p-Mentha-1,8-dien-7-al. Covance Laboratories Ltd. Study no. 8221207. May 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.
- Chen T, Harrington-Brock K and Moore MM, 2002. Mutant frequency and mutational spectra in the tk and hprt genes of N-ethyl-N-nitrosourea-treated mouse lymphoma cells. *Environmental and Molecular Mutagenesis* 39, 296-305.
- Eder E, Scheckenbach S, Deininger C and Hoffman C, 1993. The possible role of alpha,beta-unsaturated carbonyl compounds in mutagenesis and carcinogenesis. *Toxicology Letters* 67, 87-103.
- EFFA, 2012. Submission by the European Flavour Association to the European Food Safety Authority. Flavouring Group Evaluation 19 Subgroup 2.2 Flavouring Substance (Flavouring Substances) of the Chemical Group 7 (Annex I of 1565/2000/EC) Structurally Related to Alicyclic Primary Alcohols, Aldehydes, Acids and Related Esters Used as Flavouring Substances. Alicyclic alpha,beta-Unsaturated Aldehydes with the alpha,beta-Conjugation in the Ring or in the Side Chain. July 2012. FLAVIS/8.161.
- EFSA (European Food Safety Authority), 2008a. Minutes of the 26<sup>th</sup> Plenary meeting of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, Held in Parma on 27 - 29 November 2007. Parma, 7 January 2008. [Online]. Available: [http://www.efsa.europa.eu/EFSA/Event\\_Meeting/afc\\_minutes\\_26thplen\\_en.pdf](http://www.efsa.europa.eu/EFSA/Event_Meeting/afc_minutes_26thplen_en.pdf)
- EFSA (European Food Safety Authority), 2008b. Statement of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on Genotoxicity Test Strategy for Substances belonging to Subgroups of FGE.19. *The EFSA Journal* (2008), 854, 1-5.
- EFSA (European Food Safety Authority), 2008c. Scientific opinion of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on List of alpha, beta-unsaturated aldehydes and ketones representative of FGE.19 substances for Genotoxicity Testing. *The EFSA Journal* (2008), 910, 1-7.
- EFSA (European Food Safety Authority), 2012. Scientific Report of EFSA on Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports. *EFSA Journal* 2012,10(11):2977. 12pp. doi:10.2903/j.efsa.2012.2977.
- Fujita H, Aoki N and Sasaki M, 1994. [Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102 (IX\*)]. Annual Report of Tokyo Metropolitan Research Laboratory of Public Health 45, 191-199. (In Japanese)

- Gry J, Beltoft V, Benigni R, Binderup M-L, Carere A, Engel K-H, Gürtler R, Jensen GE, Hulzebos E, Larsen JC, Mennes W, Netzeva T, Niemelä J, Nikolov N, Nørby KK and Wedebye EB, 2007. Description and validation of QSAR genotoxicity models for use in evaluation of flavouring substances in Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
- Hayashi M, Kishi M, Sofuni T and Ishidate Jr M, 1988. Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals. *Food and Chemical Toxicology* 26(6), 487-500.
- Ishidate Jr M, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M and Matsuoka A, 1984. Primary mutagenicity screening of food additives currently used in Japan. *Food and Chemical Toxicology* 22(8), 623-636.
- JECFA, 2002a. Evaluation of certain food additives. Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, no. 913. Geneva, 4-13 June 2002.
- JECFA, 2002b. Compendium of food additive specifications. Addendum 10. Joint FAO/WHO Expert Committee of Food Additives 59<sup>th</sup> session. Geneva, 4-13 June 2002. FAO Food and Nutrition paper 52 Add. 10.
- Kuroda K, Tanaka S, Yu YS and Ishibashi T, 1984. [Rec-assay of food additives]. *Nippon Koshu Eisei Zasshi [Japanese Journal of Public Health]* 31(6), 277-281. (In Japanese)
- Lloyd M, 2009. Induction of micronuclei in cultured human peripheral blood lymphocytes. p-Mentha-1,8-dien-7-al. Covance Laboratories Ltd. Study no. 8200450. July 17, 2009. Unpublished report submitted by ECHA to FLAVIS Secretariat.
- Lloyd M, 2012. Mutation at the hprt locus of mouse lymphoma L5178Y cells (MLA) using the microtitre(R) fluctuation technique. p-Mentha-1,8-dien-7-al. Covance Laboratories Ltd. Study no. 8250474. June, 2012. Audited draft report. Unpublished report submitted by ECHA to FLAVIS Secretariat.
- Nikolov N, Jensen GE, Wedebye EB and Niemelä J, 2007. Report on QSAR predictions of 222 alpha,beta-unsaturated aldehydes and ketones from Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
- Sasaki M, Yoshida S, Ichikawa H, Takano I, Yasuda I and Akiyama K, 1990. [Cytological activities of perilla extracts]. *Annual Report of Tokyo Metropolitan Research Laboratory of Public Health* 41, 275-281. (In Japanese)
- SCF, 1999. Opinion on a programme for the evaluation of flavouring substances (expressed on 2 December 1999). Scientific Committee on Food. SCF/CS/FLAV/TASK/11 Final 6/12/1999. Annex I the minutes of the 119th Plenary meeting. European Commission, Health & Consumer Protection Directorate-General.
- Suzuki N and Suzuki H, 1994. Induction of K-ras codon 12 mutation by saccharin sodium in cultured human cells: [Comparison with perillaldehyde-induced mutagenicity]. *Hen'igensei Shiken* 3(3), 155-160. (In Japanese)
- Suzuki H, Ichikawa H and Sasaki M, 1990. [Mutagenicity of perillaldehyde in a human cell strain]. *Annual Report of Tokyo Metropolitan Research Laboratory of Public Health* 41, 265-266. (In Japanese)

- Tayama S, Ichikawa H and Sasaki M, 1990. [Effects of perillaldehyde on induction of chromosomal aberrations and sister-chromatid exchanges (SCEs) in CHO-KI-cells]. Annual Report of Tokyo Metropolitan Research Laboratory of Public Health 41, 267-269. (In Japanese)
- Yoo YS, 1986. Mutagenic and antimutagenic activities of flavoring agents used in foodstuffs. Osaka City Medical Journal 34(3-4), 267-288.



## ABBREVIATIONS

AFC	Food additives, flavourings, processing aids and materials in contact with food
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHL	Chinese Hamster Lung cells
CHO	Chinese Hamster Ovary cells
CoE	Council of Europe
CA	Chromosomal Aberration
EFFA	The European Flavour and Fragrance Association
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridization
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
HPRT	Hypoxanthine-guanine Phosphoribosyl Transferase
ID	Identity
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNBN	MicroNucleated BiNucleate cells
MS	Mass spectra
MSDI	Maximised Survey-derived Daily Intake
NMR	Nuclear Magnetic Resonance
No	Number
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes

PHA	PhytoHaemAgglutinin
(Q)SAR	(Quantitative ) Structure Activity Relationship
RI	Replication Index
RS	Relative Survival
S9-mix	Rat Liver Metabolic Activation System
SCE	Sister Chromatid Exchange
SCF	Scientific Committee on Food
WHO	World Health Organisation